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Fibrous Scaffold Produced By Rotary Jet Spinning Technique

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ABSTRACT
Poly(L-lactic acid) (PLLA)/ poly(ε-caprolactone) (PCL) mesh was produced by Rotary Jet Spinning (RJS) process. RJS is a simple method which fabricates three-dimensional fibers by exploiting a high-speed rotating nozzle o form a polymer jet which undergoes stretching before solidification without the need of high voltage. Blend meshes were characterized by scanning electron microscopy (SEM), thermo gravimetric analysis (TGA), differential scanning calorimeter (DSC) and infrared spectroscopy Fourier transform (FTIR). SEM imagens provides information about the morphological structure, which confirmed the production of fibers using RJS. Data obtained by thermal analyzes indicated the immiscible property of PLLA/PCL blend and also the total solvent evaporation. As a preliminary in vitro assay it was investigated using Vero cells, was not found any sign suggesting cell toxicity, indicating biocompatibility. Thus, this report suggests the use of PCL/PLLA mesh as fiber scaffold substrate for tissue engineering.

Keywords: Biomaterials, blend, polymers fibers, rotary jet spinning

I. INTRODUCTION

Tissue engineering is promissory multidisciplinary sciences that involve materials engineering and life science concepts. This science approach to develop tissues and organs to restore, maintain or improve function [1,2]. Nowadays investigations are been made to reach new techniques to obtain structures (scaffolds) that biomimics in vivo morphology. In the body all tissues are organized in three-dimensional structures as functional organs and systems. The gold standard is to be able to reach this detail level of structure in laboratory. To engineer those structures different approaches are been made.

Rotary Jet Spinning (RJS) is a technology that develops by polymeric solution addition on the cylindrical rotary device, which contains four capillary flows, that upon reaching a high rotation speed creates a jet expelled through the capillaries. After solvent evaporations in the trajectory of the capillary to the collector, the formation and retraction of the fibers occurs. This process uses centrifugal force to promote the elongation of the fibers, therefore does not require conductive polymer solutions, and since it does not uses electrostatic force as electros pinning techniques. An important point that can highlight the RJS is the high production rate comparing with the method most used electrospinning [3].

The production of biomaterial fibers has attracted much attention due the high number of applications, as well as its flexibility to adapt the composition and structure for a particular application [4]. Polymeric nanofibers have been used as scaffolds for use in cartilage, bone, arterial and nerves tissue engineering's [5]. Among the different polymeric used in the scaffolds production, the synthetic aliphatic polyesters are with great importance. Poly (lactic acid) (PLA), poly (glycolic acid) (PGA), poly (caprolactone) (PCL) and their copolymers are biocompatible and biodegradable, and also, presents distinct degradation rates [6]. PLA can presents as three different stereo chemical forms: poly (l-lactide) (PLLA), poly (d-lactide) (PDLA), and poly (dl-lactide) (PDLLA), PLLA presents a high melting temperature, due the semi crystalline structure [7]. The degradation rate is correlated with the molecular weight, purity, pH, crystallinity, presence of terminal carboxyl or hydroxyl groups, temperature, water permeability and additives acting as catalytically, therefore the degradation rate may lead more than three years to a complete resorption in vivo [8]. Poly (caprolactone) (PCL), biocompatible polymeric, with a high mechanical properties, therefore is quite common the use in bone tissue engineering [9]. The degradation rate is slowly than PLLA, it is around ~2-3 years due it high crystallity and hydrophobic nature. The great advantage of these polymers is in its forms of degradation that occur by hydrolysis of its ester bonds, so the products produced by them are absorbed by the body [10].

The choice of PLLA and PCL polymer was made by: i) are approved by the Food and Drug Administration (FDA); ii) are commonly used in tissue engineering [7-8]; iii) low cost; and iv) can successfully be blended to improve properties. The main goal of using a blend instead of single polymer is to increase properties requirements, e.g. PLLA is responsible to increase the rate of degradation and the PCL provides more flexibility to the biomaterial. Our hypothesis was to obtain fibers scaffold by RJS using different compositions of PCL with PLLA.

II. EXPERIMENTAL

2.1 Materials

PLLA molecular weight 177.500 g/mol was synthesized and provided from research group (PUC/SP Sorocaba), as described [11]. PCL molecular weight 70.000-90.000 g/mol was purchased from Sigma Aldrich. The solvents were chloroform [$CHCl_3$, 99%] from Merck (Germany) and acetone [$(CH_3)_2CO$, 99.5%] form Synth (Brazil).

2.2 Solutions preparation

Different ratios of PLLA and PCL (3:1, 1:1 and 1:3, w/w) were dissolve in mixture of chloroform/acetone (100:0, 80:20, 0:100, v/v) to prepare the solutions at a concentration of 6 wt.%. The mixtures were stirred for 2h for full homogenization. For membrane samples, it was used petri dish and was dried at ambient temperature. For solutions for RJS technique, it was used initially chloroform as the main solvent, and after the dissolution it was added acetone to a final proportion of 80:20 v/v. In was stirred for 12 hours before the use at RJS. The solutions were prepared with a total volume of 20 ml, which at constant speed fed lead to approximately 1 to 2 minutes for the fibers production.

2.3 Characterizations

Surface morphology of the meshes were characterized using Zeiss EVO MA-15 (operating mode, high vacuum, secondary electron SE detector) - the samples were gold coated, using the machine Sputter Coater (Bal-Tec SCD 050) with 40 mA, 5 x10 -7 Pa for 200 s. Fibers dimensions were measure using Image Tool software (UTHSCSA- Version 3.0); the average value was determinate using the measurements of 50 fibers of the same sample. The statistical analysis of the distribution of fiber diameter was performed using Statistic 7.0 software. Thermal properties of samples were characterized by DSC 200F3 Maia from NETZSCH differential scanning calorimeter analyzer. The method for dynamic testing was performed under an atmosphere of liquid nitrogen 50 ml / min and two scans temperature in the range of: i) first scan from 25 °C to 210 °C at a heating rate of 10°C/min and maintained at that temperature for 5 minutes; ii) second scan it was cooled to -100 °C at a rate of 10 °C/min for 5 minutes and heated again to 210 °C at a rate of 10 °C/min. The thermal degradation data was obtained by the equipment STA409C (NETZSCH) as charts mass (weight) vs. temperature (°C). The samples were heated in the range of 25 to 600 °C at a heating rate of 10 °C/min flow of 60 ml/min with a nitrogen atmosphere. The chemical changes with the different compositions were studied using Infrared Spectroscopy Fourier transforms (FTIR) Thermo Scientific - Nicolet 6700. The samples spectra were performed using average wavelength scan of 675 a 4000 cm^{-1}.

2.4 Cell Culture

Fibroblastic cell line- Vero cells- were used in this research, it were obtained from the Adolfo Lutz Institute, São Paulo, Brazil, and established from the kidney of the African green monkey (*Cercopithecus aethiops*). The cells were cultured in 199 medium (Lonza Group Ltd, USA) supplemented with 10% fetal calf serum (FCS, by Nutricell Nutrientes Celulares, Campinas, SP, Brazil) with a temperature about 37°C. Vero cells are recommended for studies of cytotoxicity and cell-substratum interactions with biomaterial researches [12,13]. The concentration of $1x10^5$cells/ml was inoculated on 24 well culture plates (Corning) on the different biomaterial compositions. After 120 hours of culture, the samples were fixed with paraformaldehyde 4% (in phosphate buffer 0.1M, pH 7.2) or ethanol/acetic acid (3/1) and stained with Cresyl Violet (CV) or Toluidine Blue (TB) at pH 4.0, respectively. We used as control cells cultured on culture plate. All experiments were made in triplicate. Through the Olympus IX-50 inverted microscope were obtained images of samples.

Also, it was performed indirect cytotoxicity assay. The extracts was obtained by added material samples in 199 medium (Lonza) with 10% FCS (Nutricell) at a final concentration of 0.2 g/ml and then incubated at 37ºC for 24 h. After this period, the medium was harvested and the materials were discarded. Vero cell suspension containing $1x10^5$cells/ml in 199 medium with 10% FCS were transferred to a 96-well culture plate (Corning Co., Cambridge, MA, USA) and cultured for 2 h at 37°C. After this incubation time, the medium was removed and the cells were incubated with biomaterial extracts. The cells were cultured by 24h in these culture conditions, according to Mosmam's Methodology. The wells were washed twice with 0.1M phosphate buffered saline (PBS)

pH 7.4, at 37°C and incubated with 100µL of 199 medium FCS-free. The assay mixture (10µL per well) containing 5mg/mL of 3-(4,5-dimetiltiazol-2-il)-2,5 diphenyl tetrazolium bromide (MTT, Sigma-Aldrich/USA) was added to each well and incubated in the dark at 37°C. After 4h, SDS (Sigma) was added to each well and 12 hours later, cells were quantified at 540nm by using multi scan micro plate reader.

2.5 Statistical analyses

The results are expressed as mean ±ST. Comparisons between groups were made using ANOVA, followed by Tukey's correction factor for multiples comparisons as a posthoc test.

III. RESULTS AND DISCUSSION

3.1 Morphology

ECM structural organization is important consideration to design biologically recognizable and acceptable biomaterials by the cells [1]. Our hypothesis was to use RJS to obtain polymeric fibers scaffold for tissue engineering. Rotary jet spinning is a simple and easy process, which the polymer solution is continuously fed into the reservoir at a rate sufficient to maintain a constant hydrostatic pressure and continuous flow rate. This process has as main advantage comparing to electrospinning the use of centrifugal force to promote the elongation of the fibers and the absence of electrostatic force.

Compositions with more PLLA or equal proportions to PCL had fibers without beads formation, which may be attributed by the solvent evaporation and ejection of the jet velocity. Beads formation was clearly observed on the 1:3 PLLA/PCL sample, beads can be formed possibly due to jet instability caused by surface tension and / or viscosity of the solution [3]. Different diameter of the fibers was produced from the rotary jet spinning, demonstrated in Fig. 1. The diameter average of the fibers of blend 3:1, 1:1 and 1:3 w/w were 12100 ± 5294, 8002 ± 5051, and 646 ± 480 nm, respectively. It is important to note that it was used the same solvent concentration by final polymeric weight, however was not considerated the MW of each polymer.

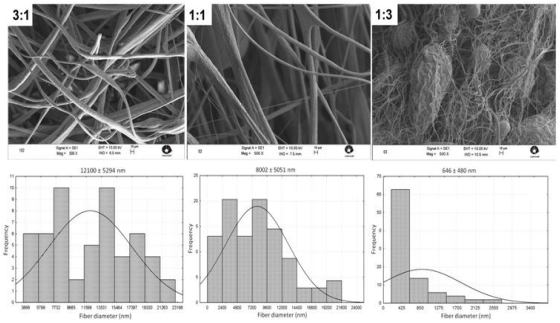

Figure 1: Morphology of PLLA/PCL blends: SEM images and histograms of 3:1, 1:1, 1:3 PLLA/PCL samples.

3.2 Differential scanning calorimeter (DSC)

Fig. 2 shows the results obtained by DSC analysis of pure polymer (pellets) and different concentration PLLA/PCL blends. The samples of PLLA and PCL showed characteristic of semicrystalline polymers results, since the DSC curves showed the presence of Tg, melting and crystallization peaks in the first and second heating.

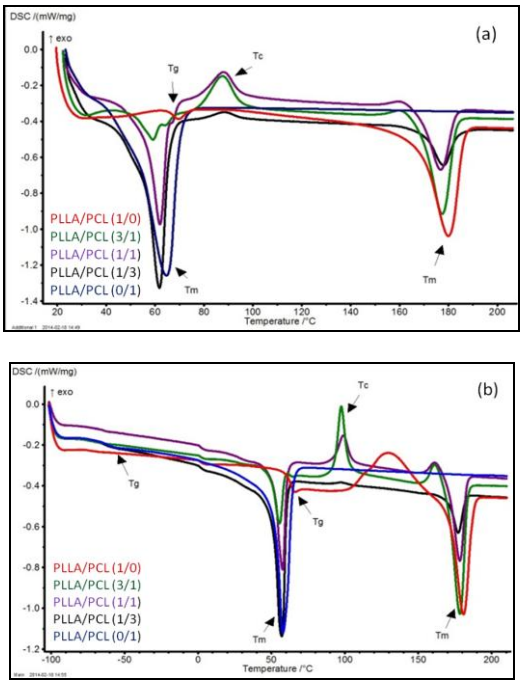

Figure 2: DSC graphics: a) first heating and b) second heating. Red and blue lines pure polymers PLLA and PCL; green, purple and black lines 3:1, 1:1 and 1:3 PLLA/PCL blends, respectively.

The first heating provides data about melting peak (Tm) and glass transition (Tg) temperatures, two melting peaks were observed at around 176-178°C and 58-64 ºC for all the blends samples, which corresponded to the melting points of PLLA segment and PCL segment, therefore each peak is more accented correlated to the sample composition. The little decrease in both Tm comparing to homopolymer of PLLA and PCL meant that the crystallization of the segments were limited by one another.

The crystallization temperature (Tc) of the PLLA and PCL pellets samples were around 102 ºC and 18 ºC, respectively. Indeed all the samples with PCL addition demonstrated lower Tc. PCL has a faster crystallization than PLLA, resulting to small and defective crystallites during the fiber creation [14]. PCL/PLLA compositions had an alteration at the degree crystallization, as expected

due PCL crystals domain and disturbs the PLLA crystalline structure. The second heating verified the Tm peaks, which were distinct around 56 ºC and 176 ºC, as PCL and PLLA phases. Also, is possible to observe the Tg of the samples PLLA around 60 ºC and for PCL -66 ºC, compatible with the literature [15,16].

3.3 Thermo gravimetric Analysis (TGA)

Table I demonstrated the results of the onset temperature of thermal degradation (Tonset) of the different samples. All the samples: PLLA, PCL and the blends showed values of the initial degradation as temperature of 340 ° C and 387 ° C respectively. The values of mass loss for the two polymers are close to literature values [15,16].

Table I Analyze TGA- Temperature of the initial degradation of the PLLA/PCL different compositions and pure polymers.

Composition PLLA/PCL [%]	**PLLA**	**PCL**
	Ti [ºC]	**Ti [ºC]**
1:0	340	-
3:1	341	386
1:1	345	-
1:3	338	390
0:1	-	387

The samples showed a similar behavior, it was observed a slight temperature variation of the onset of degradation, due to the blend composition (Table 1). The 3:1, 1:1 and 1:3 PLLA/PCL compositions had an initial weight loss at 341, 345 and 338°C, respectively, and for 3:1 and 1:3 PLLA/PCL a second at 386 and 390°C respectively, due the PCL presences. This data confirm the immiscibility of the blends.

3.4 Infrared Spectroscopy Fourier transforms (FTIR)

Fig. 3 demonstrate the spectra of the samples: pure polymers (PLLA and PCL) and the different blends (3:1, 1:1 and 1:3 PLLA/PCL) with a wavelength range of 3100 -2750 cm^{-1} and 2000-675 cm^{-1} in order to identify the changes in the chemical structures of the fibers.

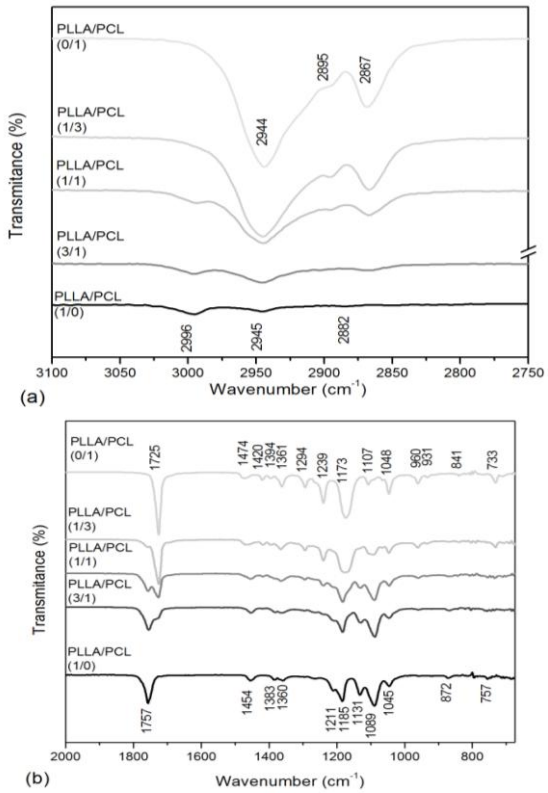

Figure 3: Spectrum of the different compositions: (a) high wavelength, 3100 to 2750 cm-1 and (b) lower wavelength 2000 to 675 cm-1.

PCL spectrum showed a symmetric stretching (CH_2) (νCH_2), characteristic of the band 2867cm^{-1}, also exhibited the bands at 2955-2925cm^{-1} commonly known for the asymmetric stretching of the CH_2 (νCH_2) [14]. The spectrum with lower wavelength presents peaks with high magnitude, 1728 cm^{-1} due the C=O bonding; 1471 cm^{-1} as CH_2 scissors; and 1176 cm^{-1} corresponding to the symmetrical stretching COC (νCOC).

In the pure PLLA spectrum was possible to observe the principals peaks: 2996 cm^{-1} corresponds to alkaline stretch (C-H), 2947 cm^{-1} due CH_3 symmetric stretching (νCH_3) and 2882 cm^{-1} corresponds CH stretching (νCH).[21] The PLLA demonstrate peaks more intense with lower wavelength: 1757 cm^{-1} due axial stretching of the carbonyl, νC = O; 1454 cm^{-1} belonging to the asymmetrical stretching CH_3; and 1185cm^{-1} which corresponds to the axial deformation of the COC bond, in the case of the polymeric structure behaves as a band COC complex ethers (νCOC) with asymmetric axial deformation [14].

The presence of the PLLA and PCL at the blends was confirmed by the peaks 2996 cm^{-1}, 2945 cm^{-1} responsible both for the C-H stretching, 1747 cm^{-1} due the C=O bonding and at 1184 cm^{-1} corresponds to the C-O bending.; also, it was demonstrated possible relationship between the

peak intensity and the polymeric concentration [17]. The concentration with high percent of PCL demonstrates magnitude of the mains peaks of PCL, decreasing with the blends with lower concentration.

3.5 In vitro assay

PLLA/PCL blend demonstrates as an important composition for tissue engineering, it combines the high value of mechanical properties from PCL with the decomposition rate and hydrophilicity property from PLLA. Due those characteristics, this blend is very investigates in different tissue engineering applications. Our hypothesis was to produce PLLA/PCL mesh for bone tissue engineering using RJS, to prove we produced different PLLA/PCL compositions using chloroform and chloroform/acetone as solvents. However, since the fast production rate of RJS, it was investigated the solvents presence in the final product. FTIR data indicates the absence of residuals solvents, however to investigate the cellular behavior with this compositions, it was produced a study with Vero cells with 2 and 5 days of culture.

Indirect in vitro assay was performed to evaluate the biocompatibility of the compositions. 24 hours of culture was used to investigate the behavior of Vero cells on different extracts. Showed in Fig. 4b-c samples presented data compatible with the negative control: 71.55±4.06, 76.97±5.32 and 74..02±4.36 of cell viability % for 3:1, 1:1 and 1:3 PLLA/PCL, respectively.

Carbohydrates and proteins cytochemical evaluations were performed. Initially, to observe the growth of the cells it was staining with blue toluidine (TB) and crystal violet (CV) for 5 days of culture Fig. 4a. TB staining is a basophilic dye that, in pH 4.0, is used for cytochemistry detection for DNA, RNA and glycosaminoglycans [18,19,20]. Basophily indicates that cells exhibit a great amount of RNA, indicating cell activity. Similar results were observed in other bioresorbable polymers, in membranes and fibers form, such as chitosan / poly (ε-caprolactone) [21]. CV is a dye used to show cellular morphology. We observed, in all samples studied, spreading cells on substrates, usually with decondensed chromatin and evident nucleolus. A confluent cell layer can be seen on various polymers studied, indicating that the cells proliferate on the samples. Indeed, after 5 days of culture was able to investigate the effects of the biomaterials on animal cells, indicating the possibility of different applications due the compatible surface, which is one of the prerequisites for implantations.

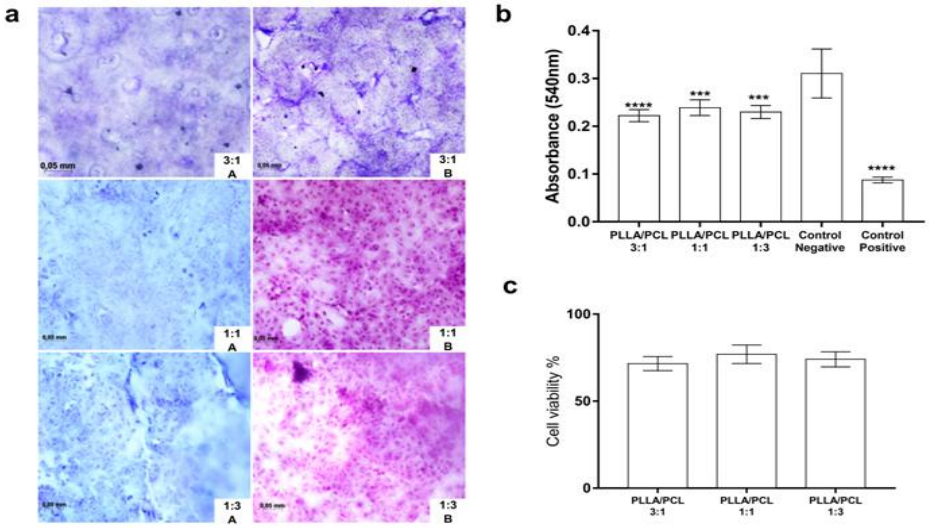

Figure 4: In vitro data a) Images after 120 hours of Vero culture 3:1,1:1 and 1:3 PLLA/PCL samples; columm A- TB staining and B- CV staining. B) Absorbance data with 24 hours of culture, *** indicates statistically differences from negative control and groups, $p<0.05$. C)Cell viability % with 24 hours of culture.

IV. CONCLUSIONS

PLLA/PCL samples demonstrated distinct morphologies, depending of the composition it modified the viscosity, which lead to an instable jet creating beads. Indeed the samples without the beads formation presented fibers with a porous surface and interconnected structure.

The immiscible compositions of PLLA/PCL were confirmed with DSC by the Tg, Tc and Tm data. Each composition showed the maintaining characteristics of the polymers, which provide the immiscibility of the blends. Indeed the thermographic analysis demonstrated a

comparative manner of the decomposition behavior of the different compositions compared to pure polymers. It was possible to observe the temperature of mass lost of the blends were very close to the initial degradation temperature of pure polymers. FTIR analysis confirmed the absence of solvents in the samples, and the correlated chemical between the polymers. The main amount of solvent is evaporated during the jet formation and solidification. In vitro data demonstrated the viability to use fibers scaffolds for tissue engineering, Vero cells showed non-apoptosis, due the biocompatible compositions. As well, cells were observed on fibers showing the adhesion surface.

Our study`s findings demonstrate that RJS is able to obtain fibers with different compositions and PLLA/PCL fibers scaffolds appears as potential scaffolds to use as cell grown substrate for tissue engineering science.

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